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APPLICATION FOR UNITED STATES PATENT

Inventors: BERNARD MOSS
MICHAEL MACKETT
GEOFFREY L. SMITH

Title: PROCESS FOR USING POXVIRUSES AS VECTORS FOR EXPRESSION OF FOREIGN GENES

Abstract of the Disclosure

Methods and compositions are provided for the use of vaccinia virus or other poxviruses as vectors for expression of foreign genes. Expression of foreign genes is obtained by combining vaccinia virus transcriptional regulatory sequences with uninterrupted foreign protein coding sequences *in vitro* to form a chimeric gene. The chimeric gene is flanked by DNA from a non-essential region of the vaccinia virus genome to provide sites for *in vivo* homologous recombination. These steps are facilitated by the construction of plasmids that contain multiple restriction endonuclease sites, next to the vaccinia virus transcriptional regulatory sequences, for insertion of any foreign protein coding sequence. Transfection procedures are used to introduce the DNA into cells infected with vaccinia virus where homologous recombination results in the insertion of the chimeric gene into a non-essential region of the vaccinia virus genome. Infectious vaccinia virus recombinants are distinguished or selected by expression of the foreign gene, loss of activity of a vaccinia virus gene, or by DNA·DNA hybridization. Expression of the foreign gene is obtained by infecting cells or

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animals with the recombinant vaccinia virus. Examples are provided to show expression of prokaryotic, RNA virus and other DNA virus genes in vaccinia virus recombinants.

Background of Invention

Recombinant DNA technology has made it possible to express genes of one organism within another. The prior art shows that several virus groups including the papovaviruses, papilloma viruses, adenoviruses, and retroviruses have been employed as eukaryotic molecular cloning and expression vectors. The relatively small sizes of these virus genomes have facilitated the *in vitro* construction of recombinant DNA molecules. However, they generally exhibit a limited host range, provide severe limitations on the amounts of DNA that can be accomodated and suffer loss of infectivity upon insertion of foreign DNA. Although genetic engineering of larger viruses, such as poxviruses, is more difficult, such vectors could have the advantage of greater capacity and potential of retaining infectivity in a wide range of host cells. For poxviruses such as vaccinia virus, such recombinants may lead to the development of live virus vaccines.

Since vaccinia virus is the best studied member of the poxvirus group, it will be described here. Vaccinia virus has a very broad host range *in vitro* and *in vivo* and has been used world-wide as an effective vaccine against variola, a related poxvirus that causes smallpox. Vaccinia is a large virus containing a linear double-stranded DNA genome with a molecular weight of about 122 million, equivalent to more than 180,000 base pairs. The virus uses its own enzymes for transcription and replication within the cytoplasm of infected cells. Nucleotide sequence data indicate that the transcriptional regulatory signals encoded in the vaccinia virus genome are distinct from those used by eukaryotic cells. The invention described here takes into account both the large size of the poxvirus genome and its unique transcriptional regulatory signals.

Prior Art Statement

References which relate to the subject invention are Venkatesan, Baroudy and Moss, Cell 125:805-813 (1981); Venkatesan, Gershowitz and Moss, J. Virol. 44:637-646 (1982); Bajszar, Wittek, Weir and Moss, J. Virol., in press; Weir and Moss, submitted for publication; Moss, Winters and Cooper, J. Virol. 40:387-395 (1981); Panicalli and Paoletti, Proc. Natl. Acad. Sci. USA 79:4927-4931 (1982); Mackett, Smith and Moss, Proc. Natl. Acad. Sci. USA, in press (1982); Cohen and Boyer, U.S. Patent 4, 237, 224.

Utility Statement

The present invention provided infectious poxvirus recombinants that express foreign genes and a general process for producing the same. When the foreign gene is an appropriate one from a pathogen, the recombinant poxvirus can serve as a live vaccine. Some examples include DNA genes or DNA copies of RNA genes from hepatitis B virus, hepatitis A virus, hepatitis non-A, non-B virus, influenza virus, herpesvirus, cytomegalovirus, adenoviruses, parvoviruses, foot and mouth disease virus, poliovirus, measles virus, rabies virus, coronaviruses, coxsackieviruses and pathogenic bacteria, rickettsia, protazoa, and metazoa. In addition, cells infected with poxvirus recombinants are used to prepare the foreign gene product.

Summary of Invention

In considering the development of vaccinia virus or other poxviruses as infectious expression vectors, the following biological characteristics of these agents were taken into account: evidence that vaccinia virus has evolved its own transcriptional regulatory sequences; its large genome size; and lack of infectivity of isolated viral DNA.

Efficient expression of foreign DNA was obtained by forming chimeric genes consisting of a vaccinia virus transcriptional regulatory sequence and an uninterrupted protein coding sequence of a foreign gene. The vaccinia virus transcriptional regulatory sequence consists of a DNA segment preceding and including the site at which RNA synthesis begins and will be referred to as a promoter. The foreign gene protein coding sequence includes the site corresponding to initiation of translation and will be referred to as the foreign gene. By using the translational initiation site of the foreign gene, codon phasing and potential problems associated with the biological activity of fusion proteins are avoided. The chimeric gene is flanked by DNA from a known non-essential region of the vaccinia virus genome that will ultimately allow homologous recombination to occur. To provide a general method of expressing foreign genes, plasmids were constructed that contain multiple restriction endonuclease sites next to the vaccinia virus promoter and that contain the flanking vaccinia virus DNA as well as the plasmid origin of replication and antibiotic resistance gene. The plasmids are then cleaved with an appropriate restriction endonuclease to form ligatable termini and a foreign gene with complementary termini is ligated next to the vaccinia virus promoter. The plasmid containing the chimeric gene and flanking vaccinia virus DNA is used to transform bacteria and then purified from the transformed bacteria.

The plasmid containing the chimeric gene flanked by vaccinia virus DNA is then used under transfecting conditions to transfect cells that have been infected with vaccinia virus or another compatible poxvirus. Homologous recombination and replication are allowed to occur. The chimeric gene is inserted into the vaccinia virus genome at the position specified by the flanking DNA used. It

is important to use flanking DNA from a non-essential region of the genome so that infectivity will not be destroyed. By using segments of the vaccinia virus thymidine kinase (TK) gene as flanking DNA, the chimeric gene can be inserted into the thymidine kinase gene within the vaccinia virus and thereby interrupt its function.

Recombinant vaccinia virus is distinguished from the original virus by a variety of methods. If the flanking DNA was from the TK gene of vaccinia virus, then virus lacking TK activity can be selected. Alternatively, virus can be distinguished either by DNA·DNA hybridization, by detection of the product of the foreign gene, or by other selective procedures.

Description of Invention

The process of this invention will be divided into the following stages:

I. Preparation of plasmid vector containing vaccinia virus promoter, sites for insertion of a foreign gene, and vaccinia virus DNA flanking sequences.

II. Preparation and insertion of foreign gene into the plasmid vector to form chimeric gene.

III. Transfection of cells with plasmid containing chimeric gene.

IV. Isolation of recombinant vaccinia virus and detection of foreign gene product.

V. Infection of susceptible cells or animals with vaccinia virus recombinants.

I. *Preparation of plasmid vector containing vaccinia virus promoter, sites for insertion of a foreign gene, and vaccinia virus DNA flanking sequences.*

The vehicle used to assemble the insertion vector may be any convenient plasmid, cosmid or phage. Examples of plasmids are pBR322, pBR325, pBR327, pBR328, pUC7, pUC8, or pUC9. The vaccinia virus DNA segment used to promote transcription of the foreign gene contained nucleotide sequences preceding and including the start site of an RNA. The nucleotide sequence and a precise transcriptional map of this region was needed for application of this method. When a convenient restriction endonuclease site preceded the RNA start site and another occurred after the RNA start site but before the first ATG or translational initiation codon, the promoter segment was excised by restriction endonuclease digestion and isolated by standard methods such as agarose gel electrophoresis. When a convenient restriction endonuclease site was not available, it was necessary to use other methods such as cleaving beyond the desired site and removing extra nucleotides with an exonuclease such as Bal31. The promoter segment directly or after modification of its ends was ligated to a plasmid that had been cleaved with a restriction endonuclease to provide compatible ligatible termini. Ligation of cohesive or blunt ends followed standard procedures. Additional restriction endonuclease sites were placed next to the promoter by inserting the promoter into a plasmid such as pUC9 that already has multiple insertion sites, however ligation of synthetic polynucleotides should also be possible. The plasmid containing the promoter was used to transform bacteria and then purified. Restriction endonucleases were used to cut out the promoter with adjacent restriction endonuclease sites and the DNA fragment was purified using conventional methods.

The DNA used to flank the promoter and added restriction endonuclease sites was derived from a non-essential region of the vaccinia virus genome. Examples of such non-essential regions include the thymidine kinase gene and a region of at least 9,000 base-pairs (bp) that is proximal to the left inverted terminal repetition. DNA containing the non-essential region was excised by restriction endonuclease cleavage and purified by agarose gel electrophoresis or other conventional methods. The segment was then ligated to plasmid DNA that had been cleaved by a restriction endonuclease to give complementary ligatable termini. The plasmid containing the vaccinia virus DNA was used to transform bacteria and then purified. An appropriate restriction endonuclease was used to cleave the non-essential segment of the vaccinia virus DNA within the plasmid so that it could be ligated to the previously isolated promoter fragment. In this manner or by variations of this procedure, a plasmid was obtained that has a vaccinia virus promoter with adjacent restriction endonuclease sites flanked by a non-essential segment of vaccinia virus DNA. Since this plasmid retained the plasmid origin of replication and antibiotic resistance gene, it was used to transform bacteria and replicated.

II. Preparation and insertion of foreign gene into plasmid vector to form a chimeric gene.

A segment of DNA containing a foreign gene or a cDNA copy of a foreign gene was obtained. The DNA segment was cleaved with restriction endonucleases at a site preceding the translational initiation codon and distal to the end of the protein coding sequences. When appropriate sites were not present, then it was necessary to cleave beyond the desired site and use an exonuclease such as Bal31 to remove extra nucleotides. For optimal expression,

the first ATG in the segment was used to initiate translation of the desired gene. Since there is no evidence for splicing of vaccinia virus RNAs, continuous protein coding sequences was used.

The plasmid constructed in part I of this section was cleaved at a restriction endonuclease site next to the promoter. The protein coding segment of the foreign gene was ligated directly to the promoter when it had complementary termini or after modification of its ends. The plasmid was used to transform bacteria and then purified. When the foreign gene was insertable in more than one orientation, it was necessary to analyze by restriction endonuclease digestion and gel electrophoresis or nucleotide sequencing to check that the proper one was obtained. The desired plasmid had the promoter adjacent to the start of the foreign gene.

III. Transfection of cells with plasmid containing chimeric gene.

Plasmids containing chimeric genes flanked by DNA from non-essential regions of the vaccinia virus genome were used to transfect cells that were already infected with vaccinia virus. The chimeric gene was inserted into the vaccinia virus genome by homologous recombination. Typically, confluent monolayers of CV-1, BSC-1, TK^{-}143, or other cells in bottles with a 25 cm^2 bottom surface area were infected with 0.01 to 0.05 plaque forming units (pfu) per cell of vaccinia virus. Approximately 1 µg of plasmid DNA with or without 1 µg of vaccinia virus DNA and 20 µg of calf thymus DNA or other carrier DNA was mixed in 1 ml of 0.1% dextrose , 0.14 M NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 20 mM Hepes, (pH 7.05) and precipitated by addition of $CaCl_2$ to a final concentration of 125 mM. The mixture was agitated gently and allowed to remain at room temperature for about 45 min. Two hr after infection, 0.8 ml of the fine suspension

was added to an infected monolayer from which medium had been removed. After 30 min, 8 ml of Eagle or other tissue culture medium containing 8% fetal bovine serum was added to each bottle and the incubation was continued at 37°C for 3.5 more hr. At 6 hr after infection, fresh medium containing 8% fetal bovine serum was added and incubation was continued for 48 hr. At this time, the infected cells were scraped off the bottle, centrifuged, resuspended in tissue culture medium and homogenized to break the cells and liberate virus.

IV. Isolation of recombinant vaccinia virus and detection of foreign gene product.

Virus from transfected cells consisted of a population of which only a small percentage were recombinants. A variety of selective and non-selective methods were used to isolate these recombinants.

Selective procedures depended on the ability of recombinants to replicate under conditions that inhibited the original virus. One selective method involved the inactivation of the vaccinia virus TK gene. This was achieved by using DNA from the vaccinia virus TK gene to flank the chimeric gene. When homologous recombination occurred, the chimeric gene was inserted into the TK gene of virion DNA and the recombinants exhibited a TK negative (TK^-) phenotype. Selective conditions for isolation of TK^- vaccinia virus was achieved by plaquing the virus in monolayers of TK^- negative cells such as TK^-143 cells with 25 μg/ml of 5-bromodeoxyuridine (BUdR) in the 1% low melting agar overlay. After 48 to 72 hr at 37°C in a 5% Co_2 humidified atmosphere, plaques were detected by staining with 0.005% neutral red. Typically, more than 30% of the TK^- plaques consisted of recombinants and the remainder were spontaneous TK^- mutants of vaccinia virus.

A second selective method be was used when TK^- cells were infected with TK^- mutants of vaccinia virus and then transfected with plasmids that contained a chimeric herpesvirus TK gene. [The TK^- mutants of vaccinia virus were obtained by infecting TK^-143 cells with vaccinia virus in the presence of 25 μg/ml of BUdR. The TK^- negative mutants were then plaqued at least 2 times in succession in TK^-143 cells in the presence of BUdR]. Recombinants expressing herpesvirus TK were selected by plaque assay on TK^-143 cells with a 1% low melting agar overlay containing Eagle medium and 8% fetal bovine serum, 100 μM thymidine, 50 μM adenosine, 50 μM guanosine, 10 μM glycine, 1 μM methotrexate. After 48 to 72 hr at 37°C in a 5% CO_2 humidified atmosphere, the plaques were detected by staining with neutral red.

Non-selective methods that depend on identification of virus plaques that contain the foreign gene were also used. In addition, such methods were used to confirm the identity of recombinants even after isolation by selective methods.

DNA·DNA hybridization was used to identify plaques formed by recombinant virus. One method was referred to as dot blot hybridization. In this procedure, virus obtained following transfection of infected cells with chimeric plasmids was plaqued on cell monolayers with a 1% agar overlay. After 48 to 72 hr, the plaques were detected by staining with neutral red. Virus within individual plaques were picked using a sterile Pasteur pipette and used to infect cell monolayers in 16 mm diameter wells of microtiter dishes. After 48 hr incubation at 37°C, the cells were scraped, lysed by three freeze-thaw cycles, and collected on nitrocellulose sheets by filtration using a micro-sample manifold (Schleicher and

Schuell, NH). The filter was washed with 100 mM NaCl, 50 mM Tris-HCl (pH 7.5), blotted three times on successive Whatman 3 MM papers saturated with (1) 0.5 M NaOH, (2) 1 M Tris-HCl (pH 7.5), and (3) 2 X SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate), baked at 80°C for 2 hr and then incubated with 5 X Denhardt's solution [Denhardt, Biochem. Biophys. Res. Commun., 23:641-646 (1966)], supplemented with 0.1 mg/ml of denatured salmon sperm DNA in 4 X SSC at 65°C for 4 hr. The foreign DNA, labeled with ^{32}P by nick translation, and sodium dodecyl sulfate (SDS) at a final concentration of 0.1% were added and hybridization continued for 12 hr. The filter was washed twice for 15 min at 65°C with 2 X SSC/0.1% SDS and then with 0.2 X SSC/0.1% SDS. An autoradiograph was made by placing the filter next to X-ray film and the presence of dark spots on developed film identified recombinant virus. Another method of DNA·DNA hybridization used was described by Villarreal and Berg [Science 196:183-185 (1977)]. In this method, a replica of virus plaques was made by placing a nitrocellulose filter directly on the cell monolayer. DNA·DNA hybridization was carried out as above and, after location of plaques containing recombinant virus, residual virus was eluted from the agar that originally overlayed the plaques.

Additional methods that depend on expression of the foreign gene were also used to identify plaques. In one case, ^{125}I-labeled antibodies to the product of the foreign gene were incubated with the cell monolayer containing virus plaques. Plaques containing recombinant virus were then identified by autoradiography. When the herpesvirus thymidine kinase was expressed, recombinant plaques were detected by incorporation of [^{125}I]deoxycytidine (1 μCi/ml) in the presence of 20 μg/ml of tetrahydrouridine from 14 to 48 hr after infection.

V. Infection of susceptible cells or animals with vaccinia virus recombinants.

After identification of vaccinia virus recombinants, 2 or more successive plaque purifications were carried out to obtain pure recombinant virus. Susceptible cells such as BSC-1, HeLa, MRC-5, or others were infected to obtain large stocks of recombinant virus. The titers of the stocks were determined by serial dilution and plaque assay.

To express the foreign gene, cells were infected with 1 to 30 pfu/cell of crude or purified virus and incubations were carried out at 37°C for up to 48 hr. The foreign gene product, depending on its nature was found in the cell culture medium or within the cells. When present in the cells, it was liberated by one of a number of methods including sonication, freeze-thawing, homogenization, or detergent treatment. The foreign protein was detected by immunological, enzymatic, and electrophoretic methods.

For infection of animals, recombinant virus was introduced intradermally, although other routes should be satisfactory. Formation of antibodies to the product of the foreign gene indicated that the foreign protein was made and was immunogenic.

EXAMPLES

In order to demonstrate the subject invention, we made several plasmids containing vaccinia virus promoters that can be used for insertion of foreign protein coding sequences to form chimeric genes. Protein coding sequences from other DNA viruses, RNA viruses and prokaryotes were inserted into the plasmids. Plasmids containing the chimeric genes then were used to transfect vaccinia virus infected cells and the recombinant virus was isolated by selective methods. Expression of the foreign genes was demonstrated in each case. Many routine procedures are described in detail in Maniatis, Fritsch, and Sambrook, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory (1982).

Example 1. Construction of plasmids pGS20 and pGS21 containing promoter from the 7.5K polypeptide gene (7.5K gene) of vaccinia virus, restriction endonuclease sites for insertion of foreign protein coding sequences, and an interrupted vaccinia virus thymidine kinase gene as flanking DNA.

(a) Isolation of 7.5K promoter DNA. A DNA fragment of approximately 275 bp that precedes and includes the RNA start site of an early vaccinia virus gene coding for a polypeptide known as 7.5K was obtained from a plasmid pAG4 [Venkatesan et al., Cell 125:805-813 (1981)]. 70 µg of pAG4 was digested to completion with 100 units of restriction endonucleases HincII and RSaI (New England Biolabs) in 50 mM NaCl, 10 mM Tris-HCl (pH 7.4), 10 mM $MgSO_4$, and 1 mM dithiothreitol (DTT) (hereafter called medium salt restriction buffer) for 2 hr at 37°C. Resulting DNA fragments were separated by electrophoresis for 1 hr at 200 volts through a 1.5% agarose gel

containing 40 mM Tris-Acetate (Tris-Ac) (pH 8.0), 20 mM sodium acetate (NaAc), 2 mM EDTA, 18 mM NaCl. The gel was soaked in 1 μg/ml ethidium bromide (EtBr) containing agarose gel buffer for 10 min. DNA fragments within the gel were visualized under long wave ultraviolet light and a gel strip containing a 275 bp DNA fragment was excised using a razor blade. DNA within this gel strip was electroblotted onto a sheet of diethylaminoethyl (DEAE)-cellulose in 40 mM Tris-Ac (pH 7.2), 20 mM NaAc, 1 mM EDTA for 45 min at 2.5 mA and eluted from the DEAE-cellulose by shaking in 1.2M NaCl, 40 mM Tris-Ac (pH 7.2), 20 mM NaAc, 1 mM EDTA for 30 min at 25°C. DEAE-cellulose was removed by centrifugation at 12,000 X g for 2 min, and DNA was precipitated from the supernatant by addition of 2 volumes of ethanol and recovered by centrifugation at 12,000 X g for 5 min.

(b) Insertion of 7.5K gene promoter into plasmid pUC9. Two μg of pUC9 DNA was digested with 5 units of restriction endonuclease HincII in medium salt restriction buffer for 2 hr at 37°C. The mixture was heated to 70°C for 5 min and DNA was extracted with an equal volume of phenol:chloroform (1:1) and recovered by ethanol precipitation. DNA was dephosphorylated at its 5' termini by incubation in 50 μl of 50 mM Tris-HCl (pH 9.0), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine with 0.1 unit of calf-intestinal alkaline phosphatase (Boehringer Mannheim) for 30 min at 37°C to prevent self-ligation in the next step. The mixture was extracted twice with equal volumes of phenol:chloroform and DNA was recovered by ethanol precipitation. 0.5 μg of linearized, dephosphorylated pUC9 DNA was ligated with 0.15 μg of the previously isolated vaccinia promoter DNA in 50 μl of 66 mM Tris-HCl (pH 7.5), 6.6 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP together with 1 unit of T_4 DNA ligase at 12°C for 15 hr. 25 μl of ligated DNA mixture was used to transform competent *E. coli* strain

JM103 and 100 μl of transformed cell suspension was mixed with 50 μl of 2% X-gal and 10 μl of 0.1M IPTG and plated onto an L broth plate containing 1.5% bacto-agar (Difco) and 50 μg/ml ampicillin. The plate was incubated at 37°C for 15 hr. White bacterial colonies were picked and grown in 10 ml of L broth containing 50 μg/ml ampicillin. Plasmid DNA was purified from 1.5 ml of bacterial cultures by the following procedure (hereafter referred to as minipreparation of plasmid DNA). Bacterial cells were pelleted by centrifugation (12,000 X g, 1 min), resuspended in 0.1 ml of 25 mM Tris-HCl (pH 8.0), 10 mM EDTA, 50 mM glucose, 2 mg/ml lysozyme (lysis solution) and incubated at 4°C for 30 min. 0.2 ml of 0.2 M NaOH, 1% sodium dodecyl sulfate (SDS) was added and the mixture incubated for a further 5 min on ice. 0.15 ml of 3M NaAc (pH 4.8) was added and the mixture incubated on ice for 1 hr followed by centrifugation at 12,000 X g for 5 min. Plasmid DNA present in the supernatant was precipitated by addition of 1 ml ethanol, recovered by centrifugation and redissolved in 0.1 ml of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (TE buffer). Plasmid DNA preparations were screened for the presence of the vaccinia virus promoter by digestion of 10% of each plasmid preparation with restriction endonucleases HindIII and EcoRI (5 units of each enzyme in medium salt restriction buffer for 1 hr at 37°C). DNA fragments were separated by agarose gel electrophoresis and visualized as described above. Plasmid preparations containing the vaccinia virus promoter were analyzed further to determine the orientation of the vaccinia promoter with respect to plasmid sequences. This was accomplished by digestion with restriction endonucleases HindIII and HincII or HincII and EcoRI (5 units of each enzyme in medium salt restriction buffer at 37°C for 1 hr) followed by agarose gel electrophoresis. A plasmid having with the vaccinia promoter reading toward the plasmid's unique BamHI restriction site was called pGS15. This plasmid was purified in large amounts by the

following procedure, hereafter called preparation of plasmid DNA. Bacteria containing the required plasmid were seeded into a 400 ml solution of M-9 medium containing 50 μg/ml ampicillin, 150 μg/ml proline, 150 μg/ml leucine, 0.8 μg/ml vitamin B_1 and grown until the optical density at 590 nm reached 0.8. Chloramphenicol was added to a final concentration of 200 μg/ml and the culture was incubated for 12 hr at 37°C. Bacteria were pelleted by centrifugation (5,000 X g, 10 min), washed in 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, resuspended in 10 ml lysis solution and incubated for 30 min on ice. 20 ml of 0.2 M NaOH, 0.1% SDS were added and the incubation was continued for 5 min on ice, followed by addition of 15 ml of 3M NaAc (pH 4.8) and a further incubation on ice for 1 hr. The mixture was centrifuged (10,000 X g, 10 min) and the supernatant was removed and recentrifuged (10,000 X g, 10 min). Plasmid DNA was precipitated by addition of 2 volumes of ethanol and recovered by centrifugation at 10,000 X g for 10 min. The DNA pellet was redissolved in 10 ml of TE buffer, the solution extracted twice with equal volumes of phenol:chloroform, the DNA recovered by ethanol precipitation and centrifugation and redissolved in 5 ml of TE buffer. 0.1 mg/ml of ribonuclease (pretreated by boiling for 10 min to inactivate deoxyribonucleases) was added and incubated for 30 min at 37°C. DNA was then precipitated by addition of NaAc (pH 7) to 0.1 M and 1.5 volumes of ethanol and recovered by centrifugation. Remaining RNA was removed from the DNA by dissolving the pellet in 0.3 M NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA and filtering it through a Sephacryl-S300 column equilibrated in the same buffer. DNA eluting in the first $A_{260\ nm}$ peak was recovered by ethanol precipitation and centrifugation. DNA was finally dissolved in TE buffer and stored at 4°C.

(c) Changing the *HindIII* site of pGS15 to an *EcoRI* site.

To enable the insertion of the vaccinia 7.5K gene promoter now cloned in pGS15 into the vaccinia thymidine kinase gene at the unique *EcoRI* site, it was necessary to change the *HindIII* site of pGS15 to an *EcoRI* site. This resulted in the vaccinia promoter and adjacent restriction sites being flanked by *EcoRI* sites. 20 µg of pGS15 DNA was cleaved with 50 units of *HindIII* restriction endonuclease in medium salt restriction buffer for 2 hr at 37°C. After extraction with phenol:chloroform, the DNA was recovered by ethanol precipitation and centrifugation. DNA termini were filled in to form blunt-ends by incubation of DNA in 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 0.5 mM DTT, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.8) with 2 units of DNA polymerase I large fragment (Boehringer Mannheim) at 37°C for 45 min. DNA was recovered after phenol:chloroform extraction by ethanol precipitation and centrifugation. Synthetic *EcoRI* linkers were phosphorylated at their 5' termini by incubation with 1 unit of polynucleotide kinase in 50 mM ATP, 66 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol at 37°C for 30 min. The phosphorylated *EcoRI* linkers were then ligated onto linearized, blunt-ended pGS15 DNA by incubation at 4°C for 15 hr in 0.5 mM ATP, 66 mM Tris-HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM DTT with one unit of T4 DNA ligase. DNA was then digested for 4 hr with 100 units of *EcoRI* in high salt restriction buffer [100 mM NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM $MgSO_4$] and fragments were separated by agarose gel electrophoresis. A 290 bp fragment was purified by electroblotting onto DEAE-cellulose and ligated to pUC9 that had been cleaved with *EcoRI* and phosphatase treated. Transformation competent *E. coli* transformed by the ligated plasmid and transformants were selected and grown and the plasmid was amplified and purified as described above. The said plasmid was termed pGS19.

(d). Insertion of 7.5K gene promoter into vaccinia virus thymidine kinase gene. Plasmid pGS8 (derived from pBR328 by insertion of the vaccinia *Hin*dIII J fragment containing the vaccinia virus TK gene into the unique plasmid *Hin*dIII site and deletion of *Bam*HI and *Eco*RI sites within the plasmid sequences), was grown and purified. 5 μg of pGS8 was digested with *Eco*RI and recovered after phenol:chloroform extraction and ethanol precipitation. 5' terminal phosphates were removed by treatment with alkaline phosphatase and the DNA was again recovered after phenol:chloroform extraction by ethanol precipitation. 0.5 μg of pGS8 DNA was then ligated together with 0.1 μg of the 290 bp DNA fragment containing the vaccinia virus promoter sequence flanked by *Eco*RI sites. This fragment had been excised from pGS19 by digestion with *Eco*RI and purified by agarose gel electrophoresis and electroblotting. Ligated DNA was used to transform competent *E. coli* cells strain HB101 and bacterial clones were screened for a plasmid containing the inserted vaccinia promoter sequence. Two such plasmids were amplified and purified; each contained the vaccinia promoter sequence but in opposite orientations with respect to plasmid sequences. The clones were termed pGS20 and pGS21. Both of these vectors have *Bam*HI and *Sma*I restriction sites for insertion of foreign genes downstream from the translocated vaccinia 7.5K gene promoter and are flanked by vaccinia DNA sequences encoding segments of the thymidine kinase gene.

Example 2. Construction of plasmids pMM3 and pMM4 that contain the promoter of the vaccinia virus thymidine kinase gene, restriction endonuclease sites for insertion of foreign protein coding sequences, and flanking DNA including part of the thymidine kinase gene.

(a) Construction of pMM1. The recent mapping and sequencing of the vaccinia virus thymidine kinase (TK) gene (Weir *et al*., 1982;

Weir and Moss, submitted for publication) allowed us to develop a strategy for isolating the TK promoter with its transcriptional initiation site but devoid of its translational start site. Inspection of the sequence showed a GTC between the transcriptional and translational start sites. If this sequence were ligated to GAC, a sequence GTCGAC recognized by several restriction enzymes would be created. This was achieved in the following manner. 25 μg of a plasmid derived from pUC9 by insertion of the vaccinia HindIII J fragment was cleaved with 50 units of ClaI (Boehringer Mannheim) for 2 hr at 37°C in 10 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 10 mM NaCl, giving a linear DNA molecule. The buffer composition was altered to 20 mM Tris HCl (pH 8.1), 100 mM NaCl, 12 mM $CaCl_2$, 1 mM NA_2EDTA and the solution was preincubated at 30°C for 10 min. Two units of the exonuclease Bal31 were added and 6 μg samples were removed at 1, 2, 5 and 10 min after addition of the nuclease. 1 μg of each of the samples was digested with 2 units of HindIII (Bethesda Research Labs) in medium salt restriction buffer at 37°C for 2 hr and the resulting fragments separated by electrophoresis on a 1% agarose gel. The time of Bal31 exonuclease digestion that gave an average size for the smallest fragment of 500 bp was chosen for further manipulation. Five μg of the 10 min nuclease digested sample was phenol extracted and ethanol precipitated. The DNA was cleaved with 10 units of HincII at 37°C for 2 hr in medium salt restriction buffer. The buffer composition was altered to 40 μM with respect to dATP, dCTP, dGTP, dTTP, 200 mM NaCl, 75 mM Tris-HCl (pH 8.8) and 10 mM $MgCl_2$ and 1 unit of E. coli DNA polymerase I (large fragment) was added and the reaction incubated at room temperature for 1 hr. The DNA was phenol extracted and ethanol precipitated. The mixture of DNA molecules with plasmid origin of replication and ampicillin resistance gene all contain GAC at one end generated by the HincII cleavage and a variable sequence at the other end. Some molecules however were

expected to have GTC between the TK gene transcriptional and translational starts at the opposing terminus. Upon ligation, these molecules would have a new *SalI* site (GTCGAC) generated. The mixture of molecules was ligated with 2 units of T4 DNA ligase (Boehringer Mannheim) at room temperature overnight in 20 mM Tris-HCl (pH 7.6), 10 mM DTT, 10 mM $MgCl_2$, 0.5 mM ATP. One μg of the ligated mixture was used to transform 100 μl of competent *E. coli* strain JM103 which were plated out on L-broth plates containing 50 μg/ml of ampicillin and grown at 37°C overnight. 144 single colonies were transferred to a master agar plate containing ampicillin. The 144 colonies were arranged in 12 groups and each group was used to inoculate L-broth, ampicillin cultures. After overnight growth, minipreparations of plasmid were prepared and 2 μg of the purified DNAs were cleaved with 2 units of *HindIII* and 2 units of *SalI* for 2 hr at 37°C in 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl. One DNA preparation contained a fragment of approximately 500 bp which would be the size of a fragment produced by *HindIII* and *SalI* if a *SalI* site had been generated after the TK transcriptional start site. The 12 colonies used to make this DNA preparation were grown singly in L-broth cultures and plasmid DNA purified and cleaved with *HindIII* and *SalI*. From one of the plasmids, a fragment of approximately 500 bp was detected by agarose gel electrophoresis. This fragment was recloned in phage M13mp8 and M13mp9 and the nucleotide sequence was determined for 100 nucleotides from both the *HindIII* and *SalI* sites according to the Sanger dideoxy chain termination method to prove that it had the desired sequences. The plasmid containing the *HindIII*- *SalI* fragment was designated pMM1 and was purified from a one liter culture of transformed *E. coli*.

(b) Construction of pMM2. 5 μg of a pUC9 derivative containing the vaccinia virus DNA *HindIII* J fragment was cleaved with 5 units of

XhoI for 2 hr at 37°C in 10 mM (Tris-HCl) pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl (high salt restriction buffer). The buffer composition was adjusted to 40 mM dATP, dCTP, dGTP and dTTP, 250 mM NaCl, 75 mM Tris-HCl (pH 8.8). One unit of E. coli DNA polymerase I (large fragment) was added and the reaction incubated at room temperature for 1 hr in order to give blunt ends. Synthetic EcoRI linkers were phosphorylated at their 5 termini by incubation with polynucleotide kinase as described previously. Phosphorylated linkers were ligated at room temperature to the blunt end DNA fragment as described previously. One mg of ligated mixture was used to transform E. coli JM103 and single ampicillin resistant colonies were picked and plasmids screened for a new EcoRI site where there had previously been an XhoI site. The plasmid with a new EcoRI site was designated pMM2.

(c). Construction of pMM3. By inserting the newly created EcoRI fragment that contains part of the TK gene from pMM2 into the EcoRI site of pMM1, a new plasmid was obtained that contains a number of restriction sites for insertion of a foreign gene coding sequence. 50 mg of pMM2 was cleaved with 75 units of EcoRI for 2 hr at 37°C in high salt restriction buffer and the 1 kb EcoRI fragment was purified by agarose gel electrophoresis and electroblotting onto DEAE paper. Five mg of pMM1 was cleaved with 5 units of EcoRI for 2 hr at 37°C as described previously. 1.5 units of bacterial alkaline phosphatase was added to the restriction digest and incubated for a further 30 min. The reaction mixture was then phenol extracted, chloroform extracted and ethanol precipitated. 0.25 mg of the EcoRI cleaved and alkaline phosphatase treated pMM1 was added to 0.5 mg of the isolated EcoRI fragment from pMM2 and the DNAs were ligated overnight. The ligated mixture was used to transform E. coli JM103 and plasmids were screened for a 1 kb EcoRI fragment inserted into pMM1 in the same

orientation as present in the vaccinia genome. The resulting plasmid designated pMM3 contains unique HincII, AccI, SalI, BamHI and SmaI sites for insertion of foreign genes next to the thymidine kinase promoter.

(d) Construction of pMM4. In order to have an EcoRI site to insert foreign genes under control of the TK promoter, it was first necessary to remove the second EcoRI site distal to the TK promoter. This was achieved in the following manner. 100 mg of pMM2 was partially cleaved with 20 units of EcoRI for 15 min at 37°C in high salt restriction buffer and the linear DNA molecule formed by cleavage at a single EcoRI site was isolated by agarose gel electrophoresis and electroblotted onto DEAE paper. 250 mg of the linear DNA molecule was incubated with 0.5 units of *E. coli* DNA polymerase I (large fragment) at 15°C for 1 hr in 40 mM dATP, dCTP, dGTP, dTTP, 250 mM NaCl, 75 mM Tris-HCl (pH 8.8). The reaction mixture was then phenol extracted, chloroform extracted and ethanol precipitated. After ligation, the plasmid was used to transform *E. coli* JM103. The resulting transformed E. coli were screened for the presence of a plasmid with the EcoRI site farthest from the promoter deleted. The plasmid, designated pMM4, contains unique HincII, AccI, SalI, BamHI, SmaI, and EcoRI sites for insertion of foreign genes.

Example 3. Formation of vaccinia virus recombinants that express the prokaryotic chloramphenicol acetyltransferase (CAT) gene.

(a) Insertion of the CAT gene into pGS21. A 770 bp DNA fragment containing the CAT gene was isolated from pBR328 by cleavage of pBR328 DNA with restriction endonuclease TaqI followed by agarose gel electrophoresis, electroblotting onto DEAE-cellulose, elution and

recovery of DNA by ethanol precipitation and centrifugation. This 770 bp DNA fragment was inserted into plasmid pUC7 as follows. pUC7 DNA was cleaved with restriction enzyme AccI, the 5' terminal phosphates were removed with calf intestinal alkaline phosphatase and the DNA was recovered after phenol:chloroform extraction by ethanol precipitation. 0.5 μg of linearized dephosphorylated plasmid DNA was ligated with 0.2 μg of the 770 bp fragment under standard conditions described above. Ligated DNA was then used to transform E. coli strain JM103 and white bacterial colonies that grew on 1.5% bacto-agar plates containing L-broth, 50 μg/ml of ampicillin, X-gal and IPTG, were picked and grown in L-broth. Mini-preparation of plasmid DNA. were screened for the presence of the 770 bp DNA fragment containing the CAT gene by digestion with BamHI and agarose gel electrophoresis. Such a plasmid was grown, amplified and purified by standard procedures as described above and called pGS29.

Plasmid pUC7 contains 2 BamHI sites closely flanking the AccI sites. Consequently, after insertion of the CAT gene into the AccI site, this gene was now excisable as a 780 bp fragment by BamHI. After BamHI digestion of pGS29, the 780 bp fragment was isolated by agarose gel electrophoresis, electroblotted onto DEAE-cellulose, eluted and recovered by ethanol precipitation.

The next step was the insertion of this BamHI DNA fragment into plasmid vector pGS21. pGS21 DNA was linearized by cleavage with BamHI, the 5' terminal phosphates were removed by digestion with calf intestinal alkaline phosphatase and DNA was recovered by phenol:chloroform extraction and ethanol precipitation. 0.5 μg of linearized, dephosphorylated pGS21 DNA was ligated with 0.1 μg of the 780 bp DNA fragment under standard conditions and the ligated DNA was then used to transform, competent E. coli cells strain HB101.

Transformed cells were plated onto an L-broth plate containing 1.5% bacto-agar and 50 μg/ml ampicillin. After incubation for 15 hr at 37°C, bacterial colonies were picked grown in L-broth containing 50 μg/ml ampicillin and plasmid DNA was purified by the minipreparation procedure. Plasmid DNA was screened for the presence of the 780 bp CAT gene BamHI fragment in the correct orientation with respect to the vaccinia promoter by digestion with BamHI or EcoRI followed by agarose gel electrophoresis. Such a clone was called pGS24 and was grown, amplified and purified as described above.

(b) Insertion of the chimeric CAT gene into vaccinia virus. A 25 sq cm monolayer of TK^-143 cells was infected with wild type vaccinia virus at 0.01 pfu/cell. A mixture of 1 μg of pGS24, 1 μg vaccinia virus DNA, and 20 μg of calf thymus DNA was precipitated with 125 mM $CaCl_2$. The fine suspension was used to transfect the cells at 2 hr after infection. After 30 min at 25°C, 7.2 ml of Eagle medium containing 8% fetal bovine serum was added and the monolayer was incubated for 3.5 hr at 37°C. The culture medium was then removed and replaced by 8 ml fresh Eagle medium containing 8% fetal bovine serum and the incubation was continued at 37°C for two days. Cells were scraped from the bottles, pelleted by centrifugation (2,000 X g, 5 min) and resuspended in 0.5 ml of Eagle medium containing 2.5% fetal bovine serum.

(c) Selection of recombinant vaccinia virus containing the chimeric CAT gene. Thymidine kinase negative vaccinia virus recombinants were selected by plaque assay in TK^-143 cells with a 1% low melting agarose overlay containing 25 μg/ml BUdR. After three days at 37°C, cell monolayers were stained with 0.005% neutral red, plaques were picked using a sterile Pasteur pipette and placed in 0.5 ml of Eagle medium containing 2.5% fetal bovine serum. After

freezing and thawing 3 times and sonication, 0.25 ml of each plaque was used to infect 16 mm diameter monolayers of TK^-143 cells. Two hr after infection, culture medium was removed and monolayers were overlayed with 1 ml of Eagle medium containing 2.5% fetal bovine serum and 25 μg/ml BUdR. After two days incubation at 37°C, cell monolayers were scraped from wells, pelleted by centrifugation, resuspended in 0.5 ml of 0.15 M NaCl, 10 mM Tris-HCl (pH 7.5), frozen and thawed 3 times and 0.1 ml was transferred to nitrocellulose using a micro filtration manifold (Schleicher and Schuell). After procedures to denature, neutralize and fix DNA to the nitrocellulose, the filter was hybridized with ^{32}P-labeled CAT gene DNA. After washing the filter, an autoradiograph was obtained. Virus recombinants showing positive hybridization to ^{32}P-CAT DNA were further plaque purified in TK^-143 cells with BUdR selection and the screening procedure for CAT DNA repeated. A clone positive for CAT DNA at this stage was then amplified once in TK^- cells with BUdR selection, once in CV-1 cells without selection and the virus titre determined by plaque assay in CV-1 cells. This virus was called vCAT24.

(d) Analysis of expression of chimeric CAT gene. CV-1 cells were infected with vCAT24 at 10 pfu/cell. After 24 hr at 37°C, cells were scraped, pelleted, resuspended in 0.2 ml of 0.25M Tris-HCl (pH 7.8) and sonicated. Cell debris was removed by centrifugation (12,000 X g, 5 min) and the supernatant was assayed for chloramphenicol acetyltransferase activity essentially as described by Gorman et al., Mol. Cell Biol. 2:1044-1051 (1982). Extracts from cells infected with vCAT24 contained an enzyme activity that acetylated chloramphenicol as demonstrated by thin layer chromatography. Extracts from both uninfected and wild-type vaccinia

virus infected cells contained no detectable chloramphenicol acetyltransferase activity.

Example 4. Construction of vaccinia virus expressing chimeric herpes thymidine kinase (HTK) gene. 50 μg of a plasmid containing a BamHI fragment including the HTK gene [Enquist et al., Gene 7:335-342 (1979)] was cleaved with 50 units of HincII and 50 units of PvuII for 2 hr at 37°C in medium salt restriction buffer. A 1.8 kb HincII-PvuII fragment devoid of the HTK transcriptional start site but containing all of its coding sequence was isolated by agarose gel electrophoresis and electroblotted onto DEAE paper. Five μg of pUC7 was cleaved with 5 units of HincII for 2 hr at 37°C in medium salt restriction buffer. 1.5 units of calf intestinal alkaline phosphatase was added and the reaction mixture was incubated for a further 30 min after which it was phenol extracted, chloroform extracted and ethanol precipitated. 250 ng of this DNA was ligated with 500 ng of the isolated HincII-PvuII HTK fragment and E. coli JM103 cells were transformed with the ligated mixture. Plasmids were isolated from the transformed bacteria and screened for the HincII- PvuII fragment inserted into pUC7. This plasmid was designated pVH4. 50 μg of pVH4 was cleaved with EcoRI and the EcoRI fragment containing the HTK gene was isolated by agarose gel electrophoresis and electroblotted onto DEAE paper. Five μg of pMM4 was cleaved with EcoRI; 1.5 units of calf intestinal alkaline phosphatase was added and the reaction incubated for a further 30 min. The reaction mixture was then phenol extracted, chloroform extracted and ethanol precipitated. 250 ng of this cleaved plasmid was ligated to 500 ng of the EcoRI fragment containing the HTK coding sequences and this ligated mixture was used to transform E coli JM103. Single ampicillin resistant colonies were screened for a plasmid containing HTK coding sequences at the EcoRI site of pMM4.

This recombinant plasmid was designated pMM20 and combines the vaccinia virus TK transcriptional regulatory sequences with an uninterrupted Herpes TK coding sequences, and the distal half of the vaccinia virus TK gene. This chimeric gene is flanked with vaccinia sequences such that homologous recombinantion would occur at the site of the vaccinia virus TK gene of wild type virus.

Human TK^-143 cells were infected with 0.01 pfu/cell of a vaccinia virus TK^- mutant (TK^-13; Bajszar et al., J. Virol., in press). The TK lesion maps between the vaccinia TK transcriptional start site and the EcoRI site in the vaccinia TK gene and hence the lesion could not be restored to wild type vaccinia TK by recombination with pMM20. One μg of calcium phosphate precipitated pMM 20 DNA was introduced by transfection into TK^-143 cells infected with TK^-13 vaccinia virus. Selection procedures described earlier for selection of TK^+ virus were used. Isolated single TK^+ plaques were plaque purified again and checked for synthesis of the herpesvirus TK. Since [I^{125}]dC is a specific substrate for the herpesvirus TK, but not for vaccinia virus or cellular TK, [^{125}I]dc is incorporated into viral DNA only if herpes virus TK is expressed. Autoradiography of cell monolayers, infected with the putative recombinant virus, in the presence of [^{125}I]dC revealed dark spots on the film corresponding to viral plaques showing that the TK^+ virus was expressing herpesvirus TK. That the herpes TK was integrated into the viral genome was shown by DNA·DNA hybridization of ^{32}P-labeled HTK DNA to blots of separated restriction digests of purified recombinant viral DNA. A further confirmation that herpesvirus TK was expressed was obtained by plaquing a recombinant virus stock in the presence and absence of 40 μg/ml bromodeoxycytidine (BCdR). Wild type vaccinia virus plaques were slightly reduced in size but the number of plaques remained constant when the media was supplemented with 40 μg/ml BCdR. However, the

titer of recombinant virus was reduced between 10 to 100-fold in the presence of 40 μg/ml BCdR, as expected if synthesis of the herpesvirus TK had occurred.

Example 5. Construction of vaccinia virus recombinants expressing chimeric vesicular stomatitis virus (VSV) N gene. 50 μg of pJS223 (a plasmid containing a cDNA copy of VSV N gene, Sprague *et al.*, in press) was cleaved with 50 units of *Xho*I for 2 hr at 37°C in high salt restriction buffer. The smaller fragment containing N gene DNA was isolated by agarose gel electrophoresis and electroblotting onto DEAE paper. Five μg of pMM3 was cleaved with 5 units of *Sal*I for 2 hr at 37°C. 1.5 units of calf intestinal alkaline phosphatase was added for a further 30 min. The reaction mixture was phenol extracted, chloroform extracted, and ethanol precipitated. 250 ng of this DNA was ligated to 500 ng of the isolated *Xho*I fragment and *E. coli* JM103 were transformed with the ligated mixture. Single ampicillin resistant colonies were screened for the presence of VSV cDNA cloned into pMM3. The chimeric plasmid containing the vaccinia promoter contiguous with the VSV cDNA was designated pMM17. Cells were infected with wild type vaccinia virus and transfected with this plasmid. After 48 hr, the cells were disrupted and TK^- virus was selected by plaque formation with BUdR in the agar overlay. Expression of the chimeric VSV N gene was shown by standard immunoprecipitation methods. Recombinant virus was used to infect cells at 20 pfu/cell and [^{35}S]methionine was added to the medium. At 6 hr after infection, a cytoplasmic extract was made from the infected cells. Rabbit anti-VSV antiserum and staph A protein were used to precipitate VSV proteins. After dissociation of the Staph A -protein complex, the proteins were analyzed on a 10% polyacrylamide gel in parallel with authentic VSV labeled proteins. After fluorography, a protein that was immunoprecipitated from cells

infected with the recombinant virus was seen to comigrate with authentic VSV N protein. This result demonstrated that the vaccinia virus recombinant expressed the chimeric VSV N gene.

Deposition of Materials

A Sample of E. coli HB101 transformed with pGS20 has been deposited on 11/30/82 at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 and has been given ATCC No. 39249.

Ins C1

C1

CLAIMS

1. A method of preparing a plasmid containing a chimeric gene composed of Vaccinia Virus ~~poxvirus~~ transcriptional regulatory sequences and protein coding sequences from a foreign gene with DNA from a non-essential region of the Vaccinia virus ~~poxvirus~~ genome flanking the said chimeric gene by:

a. preparing a plasmid containing Vaccinia Virus ~~poxvirus~~ transcriptonal regulatory sequences next to multiple restriction endonuclease sites with DNA from a non-essential region of the genome flanking said transcriptional regulatory sequences and multiple restriction endonuclease sites and

b. inserting protein coding sequence from a foreign gene into a restriction endonuclease site next to the ~~Poxvirus~~ Vaccinia Virus ~~vaccinia virus~~ transcriptional regulatory sequence.

2. The method according to Claim 1 wherein the Vaccinia Virus ~~poxvirus~~ transcriptional regulatory sequence consists of DNA preceding and including the site at which RNA synthesis starts.

Subt C2

3. The method according to Claim 1 wherein the transcriptional regulatory DNA sequence is from the vaccinia virus thymidine kinase gene or from an early vaccinia virus gene encoding a polypeptide known as 7.5K.

4. The method according to Claim 1 wherein the poxvirus is vaccinia virus.

5. The method according to Claim 1 wherein the protein coding sequence of the foreign gene includes the site corresponding to initiation of translation and extends beyond the translational termination site.

6. The method according to Claim 5 wherein the protein coding sequence is obtained from a DNA copy of a DNA gene or a DNA copy of a RNA gene.

Subt C3

7. The method according to Claim 1 wherein the non-essential region is the vaccinia virus thymidine kinase gene.

8. The method according to Claim 1 wherein the plasmid formed is pGS20, ~~pGS21, pMM3, and pMM4~~.

9. A method of inserting a chimeric gene flanked by Vaccinia Virus ~~poxvirus~~ DNA from a non-essential region contained in a plasmid into the Vaccinia Virus ~~poxvirus~~ genome by transfecting cells infected with a Vaccinia ~~poxvirus~~ so that homologous recombination occurs and the recombinant Vaccinia Virus ~~vaccinia virus~~ expresses the foreign gene upon infection of cells.

10. A method of isolating recombinant poxvirus under selective conditions in which only thymidine kinase negative virus forms plaques or only thymidine kinase positive virus plaques.

11. A method of using the recombinant poxvirus as a live vaccine.

add a' + B'